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Direct broad-range detection of alphaviruses in mosquito extracts

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Abstract

Members of the genus *Alphavirus* are a diverse group of principally mosquito-borne RNA viruses. There are at least 29 species and many more subtypes of alphaviruses and some are considered potential bioweapons. We have developed a multi-locus RT-PCR followed by electrospray ionization mass spectrometry (RT-PCR/ESI-MS) assay that uses the amplicon base compositions to detect and identify alphaviruses. A small set of primer pairs targeting conserved sites in the alphavirus RNA genome were used to amplify a panel of 36 virus isolates representing characterized Old World and New World alphaviruses. Base compositions from the resulting amplicons could be used to unambiguously determine the species or subtype of 35 of the 36 isolates. The assay detected, without culture, Venezuelan equine encephalitis virus (VEEV), Eastern equine encephalitis virus (EEEV), and mixtures of both in pools consisting of laboratory-infected and -uninfected mosquitoes. Further, the assay was used to detect alphaviruses in naturally occurring mosquito vectors collected from locations in South America and Asia. Mosquito pools collected near Iquitos, Peru, were found to contain an alphavirus with a very distinct signature. Subsequent sequence analysis confirmed that the virus was a member of the *Mucambo virus* species (subtype IIID in the VEEV complex). The assay we have developed provides a rapid, accurate, and high-throughput assay for surveillance of alphaviruses.

Keywords: Alphavirus; Biodefense; Emerging pathogen; VEEV; Venezuelan equine encephalitis; Togaviridae; Virus detection; Mucambo IIID virus; Viral diagnostic

Introduction

The genus *Alphavirus* consists of a group of enveloped, single-stranded, positive-sense RNA viruses within the family *Togaviridae*. These viruses are principally mosquito-borne and have a nearly worldwide distribution (Griffin, 2001). The genus contains at least 29 distinct, known species (some with multiple subtypes or varieties), subdivided into seven antigenic complexes, containing both New World and Old World members. New species have been described recently from mosquitoes (Travassos da Rosa et al., 2001), from fish (Villoing et al., 2000; Weston et al., 1999), and from a louse infesting the southern elephant seal (La Linn et al., 2001). Many of the alphaviruses are significant pathogens for humans and domestic animals. Clinically, disease manifests either as a mild, self-limiting febrile illness, central nervous system infection (i.e., encephalitis), or as a febrile illness with rash and polyarthropathy. New World viruses such as Venezuelan equine encephalitis virus (VEEV), Eastern equine encephalitis virus (EEEV), and Western equine encephalitis virus (WEEV) mainly cause encephalitis, whereas the Old World viruses (such as Ross River, Barmah Forest, O'nyong-nyong, Chikungunya, and Sindbis viruses) cause an arthralgia syndrome (Tsai and Monath, 2002). Getah

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14. ABSTRACT
Members of the genus Alphavirus are a diverse group of principally mosquito-borne RNA viruses. There are at least 29 species and many more subtypes of alphaviruses and some are considered potential bioweapons. We have developed a multi-locus RT-PCR followed by electrospray ionization mass spectrometry (RT-PCR/ESI-MS) assay that uses the amplicon base compositions to detect and identify alphaviruses. A small set of primer pairs targeting conserved sites in the alphavirus RNA genome were used to amplify a panel of 36 virus isolates representing characterized Old World and New World alphaviruses. Base compositions from the resulting amplicons could be used to unambiguously determine the species or subtype of 35 of the 36 isolates. The assay detected, without culture, Venezuelan equine encephalitis virus (VEEV), Eastern equine encephalitis virus (EEEV), and mixtures of both in pools consisting of laboratory-infected and -uninfected mosquitoes. Further, the assay was used to detect alphaviruses in naturally occurring mosquito vectors collected from locations in South America and Asia. Mosquito pools collected near Iquitos, Peru, were found to contain an alphavirus with a very distinct signature. Subsequent sequence analysis confirmed that the virus was a member of the Mucambo virus species (subtype IIID in the VEEV complex). The assay we have developed provides a rapid, accurate, and high-throughput assay for surveillance of alphaviruses.

15. SUBJECT TERMS
Venezuelan equine encephalitis virus, VEE, eastern equine encephalitis, EEE, detection, identification, RT-PCR, electrospray ionization/mass spectrometry, vectors

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virus has been reported to induce abortion or stillbirth in pregnant swine (Kiyomasu et al., 1991) and Highlands J virus causes dramatic decreases in egg production and mortality in domestic birds (Wages et al., 1993). Disease of salmon and rainbow trout, caused by two recently described fish alphaviruses (Villoing et al., 2000; Weston et al., 1999; Jewhurst et al., 2004), has the potential for significant economic damage to the commercial fish industry. Finally, some alphaviruses such as EEEV have the potential to be used as bioweapons (Sidwell and Smee, 2003; Vogel et al., 1997), and therefore the ability to detect any and all alphaviruses has applications for biodefense.

Several genus-specific and multiplex molecular methods have been described for the detection of various alphavirus members (Bronzoni et al., 2004; de Morais Bronzoni et al., 2005; Lee et al., 2002; Linssen et al., 2000; Pfeffer et al., 2002; Pfeffer et al., 1997; Sanchez-Seco et al., 2001; Wang et al., 2006). Here we describe a rapid, multi-locus method for the identification of New and Old World alphaviruses. RT-PCR amplicons from three viral loci were analyzed by electrospray ionization mass spectrometry (PCR/ESI-MS) and base compositions (the numbers of adenines, guanines, cytosines, and thymines) of the amplicons determined (Ecker et al., 2005; Hofstadler et al., 2005; Sampath et al., 2005). The base compositions of these three regions were used to unambiguously assign the viral subtype. The present study was performed using a collection of 36 different species and subtypes of alphaviruses. In addition, field-collected mosquito pools were analyzed. A result of this study was the discovery of an alphavirus with a unique base composition signature. Subsequent sequence analysis of the virus confirmed that the virus found in mosquitoes collected near Iquitos Peru is a relatively new *Mucambo virus* subtype IIID in the VEEV complex and is probably the same or closely related to a virus that was previously speculated to be a subtype IIID virus in the VEEV complex (Aguilar et al., 2004), which was found in a human patient living in the same region of Peru where our mosquito samples were collected. These results indicate the power of the assay to detect previously uncharacterized and emerging alphaviruses.

Results

Broad-range PCR primer pairs

Alphaviruses are an extremely variable group of viruses and pose a major challenge for most molecular diagnostic assays. A goal of this work was to develop an assay that would, in a single format, allow the detection of all of the diverse members of this group of viruses. To accomplish this, a large number of primer pairs were designed and tested (data not shown). Three primer pairs were chosen for use in the final assay format. These primer pairs are described in the top half of Table 1. Primer pair VIR966 exhibited the greatest breadth of coverage. Fig. 1 shows the alignment of this primer pair against the known sequences of several diverse alphaviruses. For each primer region, a database of expected base compositions (A, G, C, and T base counts) from all known alphavirus sequences in GenBank was generated (data not shown) and used in the identification and classification of the test isolates. Several of the isolates used in this study did not have a genome sequence record in GenBank and the base compositions for the three target amplicons were determined experimentally.

Detection of diverse alphavirus isolates

The primers shown in Table 1 were tested for their ability to amplify a collection of diverse alphavirus isolates (Table 2), including isolates from both the Old and New World members. The primer pair sequences were experimentally optimized by testing their performance against this collection of alphavirus isolates. Table 3 shows the experimentally determined base compositions for these isolates. Isolates tested showed 100% matches to expected base compositions where sequences were known for amplified regions. Primer pair VIR966 provided the broadest coverage and amplified all isolates with the exception of Una virus. A subset of target viruses did not amplify with these primers, however. Primer pair VIR2499, for instance, did not produce any amplicons from a subset of the Old World

Table 1
RT-PCR and sequencing primers

Application	Target	Primer pair number	Primer coordinates	Orientation	Primer sequence
Broad-range RT-PCR	nsP1	VIR966	151–178	F	TCCATGCTAATGCTAGAGCGTTTTCGCA
			225–248	R	TGGCGCACTTCCAATGTCCAGGAT
		VIR2499	1044–1072	F	TGCCAGCIACAITGTGIGAICAIATGAC
			1122–1149	R	TGACGACTATICGCTGGTTIAGCCCIAC
	nsP4	VIR2052	6971–6997	F	TGGCGCTATGATGAAATCTGGAATGTT
			7086–7109	R	TACGATGTTGTCGTCGCCGATGAA
VEEV sequencing	nsP1	VEE6AI	45–76	F	M13F/ATGGAGAAAGTTCACGTTGACATIGAIGIIGA
			579–610	R	M13R/GCCAAGTTCTTAAACATAAAAGGIGTIGTITC
	nsP4	VEE9AI	6819–6850	F	M13F/TCGTTTGATAAAAGTGAGGACGAIGCIATGGC
			7366–7397	R	M13R/CAGCTCTGAAAGAATACCCACTCGITTCCAIC
	E1	VEE10AI	10799–10830	F	M13F/CGCGCCGAAAACTGTGCTGTAGGITCIATICC
			11291–11322	R	M13R/TGTTTCTGGTTGGTCAGCACGTACATIGCIAC

The broad surveillance alphavirus primers were chosen by computational analysis of sequence alignments using all available GenBank alphavirus sequences. The letter "I" in the primer sequence notes the position of an inosine. The forward and reverse sequencing primers have the following sequences at their 5′ ends to facilitate the sequencing of the RT-PCR amplicons: M13F, CCCAGTCACGACGTTGTAAAACG; M13R, AGCGGATAACAATTTCACACAGG. Reference genome VEEV type ID, Strain, NC_001449.

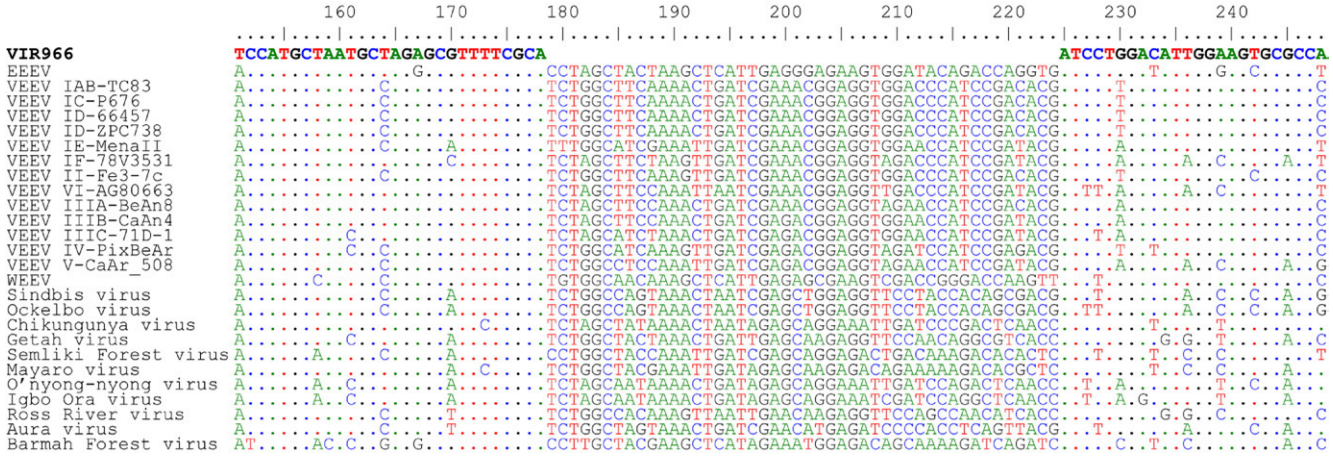

Fig. 1. Alignment of alphavirus sequences showing conservation of PCR primer regions within this viral family, flanking a region of species-specific variations. "Dots" in a column represent homology to the reference sequence above. Any variant is explicitly shown with the varying nucleotide.

viruses. Likewise primer pair VIR2052 did not amplify some of the VEEV and EEEV subtypes. Each virus strain was amplified by a minimum of two primer pairs with the exception of Una virus.

By using base compositions from two or more amplicons, most of the species and subtypes in Table 3 were clearly distinguished from each other. For instance, all the VEEV complex subtypes were distinguished from other New World viruses, including all the EEEV and WEEV complex viruses. Occasionally there was an overlap of base compositions in a single primer region between species, but there was no overlap of signatures when two or more primer regions were taken together. In addition to species level resolution, the measured base compositions were also useful in distinguishing isolates at

Table 2
Alphaviruses isolates used in this study

Virus	Strain	Origin, year	Source	Passage history[a]	GenBank accession no.
EEEV (II)	Arg-LL	Argentina, 1936	Horse	v5, BHK2	U01560, U01600, U01640
EEEV	PE-0.0155	Peru, 1996	Mosquito	Not passaged	DQ241304
EEEV	76V-25343	Brazil, 1976	Mosquito	v3, BHK2	U01564, U01607, U01647
EEEV (I)	GA97	Georgia (USA), 1997	Human	v3, BHK2	AY705240
VEEV (IA/B)	Trinidad donkey	Trinidad, 1943	Donkey	gp1, cef14, sm2, v1, BHK3	L01442
VEEV (IC)	CO951006	Columbia, 1995	Human	v2, BHK2	U55350
VEEV (ID)	V209A	Colombia, 1960	Mouse	sm2, v3, BHK1	AF004465, U55361
VEEV (IE)	68U201	Guatemala, 1968	Hamster	sm4, BHK3, v1, BHK1	U34999
VEEV (IF)	78V3531	Brazil, 1978	Mosquito	dec1, sm3, BHK1, v1, BHK1	AF004437
VEEV (II)	Everglades Fe3-7c	Florida, 1963	Mosquito	sm5, v2, BHK2	AF075251
Mucambo (IIIA)	Mucambo BeAn8	Brazil, 1954	Monkey	sm2, BHK2, v1, BHK1	AF075253
VEEV (IV)	Pixuna BeAr40403	Brazil, 1961	Mosquito	sm6, v3, BHK1	AF075256
VEEV (V)	Cabassou CaAr508	French Guiana, 1968	Mosquito	sm5, v2, BHK2	AF075259
VEEV (VI)	Ag80-663	Argentina, 1980	Mosquito	sm6, v1, BHK2	AF075258
WEEV	Fleming	California,	Human	sm5	AF109297, U60380
WEEV-AR58	CBA87	Argentina, 1958	Horse	sm1, BHK2, v1, BHK1	DQ432026
WEEV-SD83	R-43738	South Dakota, 1983	Human	sm2	NR
WEEV-ON41	McMillan	Ontario, Canada, 1941	Human	sm4	AF229608
WEEV-OR71	71V1658	Oregon, 1971	Horse	wc1, de1, sm1, v1, bhk2	U60364
Highlands J virus	B230	Florida, 1960	Blue jay	sm4, v1, BHK2	AF339476
Sindbis virus	UgMP6440	Uganda	NR	sm4,V1, BHK2	NR
Chikungunya virus	15561	Thailand	Human	gmk2, BHK1, v1	NR
Getah virus	MM 2021	Malaysia, 1955	Mosquito	sm5, v1, BHK2	AF398377
Mayaro virus	BeH256	Brazil	Mosquito	sm8, v4, BHK1	NR
Semliki Forest virus	Original	Uganda, 1942	Mosquito	sm7, v1, BHK2	NR
Una virus	CoAr43332	Colombia, 1964	Mosquito	Not from DSD	AF398381
Barmah Forest virus	BH2193	Australia, 1974	NR	sm3, v1, BHK2	U73745
Middelburg virus	SA AR749	South Africa, 1959	Mosquito	sm12, v1, BHK2	AF398374
Ndumu virus	SA AR221D	South Africa, 1959	Mosquito	sm8, V1, BHK2	AF398375
Tocora virus	PE-70009	Peru, 1997	Mosquito	From Mike Turell	AF252264

NR, not reported.

[a] Cell types: dec, duck embryo cells; sm, suckling mouse; v, Vero; gp, guinea pig; BHK, baby hamster kidney; cef, chick embryo fibroblasts; gmk, green monkey kidney.

Table 3
Base compositions of the RT-PCR amplicons generated using primer pairs VIR966, VIR2499, and VIR2052

Viral complex	Species name	Origin / subtype	Strain name	PP966	PP2499	PP2052
EEEV	Eastern equine encephalitis virus	I	Georgia (1997)	[26 27 22 23]	[23 36 28 18]	[31 31 36 41]
		II	76V-25343 (1976)	[27 25 25 21]	[24 34 26 21]	[DNP]
		III	ARG-LL (1936)	[27 25 25 21]	[24 34 26 21]	[DNP]
		Peru	PE-3.0869	[27 25 25 21]	[24 34 26 21]	[DNP]
		Peru	PE-0.0155	[27 25 25 21]	[24 34 26 21]	[DNP]
VEEV	Venezuelan equine encephalitis virus	IA/B	Trinidad donkey	[25 25 26 22]	[27 34 27 17]	[41 33 31 34]
		IC	CO951006	[25 25 26 22]	[27 34 27 17]	[41 33 33 32]
		ID	1D V-209-A-TVP1163	[25 25 26 22]	[27 34 27 17]	[41 33 33 32]
		IE	68U201	[25 27 22 24]	[27 34 26 18]	[34 34 32 39]
		IF	78V-3531	[25 24 24 25]	[25 34 26 20]	[DNP]
		II	Everglades Fe3-7c	[24 26 25 23]	[27 33 25 20]	[36 34 32 37]
		VI	AG80-663	[26 22 25 25]	[27 31 23 24]	[36 31 33 39]
	Mucambo virus	IIIA	BeAn 8	[27 23 26 22]	[27 33 26 19]	[35 32 32 40]
		IIIC	PE-24.0269	[26 25 24 23]	[26 32 28 19]	[38 27 34 40]
		IIID	PE-4.0766	[27 23 23 25]	[27 32 28 18]	[38 30 30 41]
	Pixuna virus	IV	BeAr 35645	[25 27 23 23]	[28 31 28 18]	[36 34 30 39]
	Cabassou virus	V	CaAr 508	[25 25 25 23]	[27 35 25 18]	[DNP]
WEEV	Western equine encephalomyelitis virus		SD83	[26 27 23 22]	[28 33 23 21]	[38 32 33 36]
			CBA 87/4	[26 27 23 22]	[28 33 23 21]	[38 33 32 36]
			PE-1.0433	[26 27 23 22]	[28 33 23 21]	[36 34 33 36]
			ON41	[26 27 23 22]	[28 33 23 21]	[37 33 33 36]
			OR71	[26 27 23 22]	[28 33 22 22]	[37 33 33 36]
			VR-1251 (Flemming)	[26 26 23 23]	[28 33 23 21]	[37 33 33 36]
	Sindbis virus		UgMP6440	[24 25 26 23]	[29 29 27 20]	[37 33 33 36]
	Highlands J virus		B230	[26 28 22 22]	[28 34 27 16]	[36 33 28 42]
SFV	Chikungunya virus	Thailand	15561 vaccine parent	[30 20 25 23]	[DNP]	[35 33 35 36]
	Una virus	Peru	CoAr43332	[DNP]	[DNP]	[43 31 33 32]
			PE-21026	[DNP]	[DNP]	[45 28 32 34]
			PE-10800	[DNP]	[DNP]	[44 29 31 35]
	Getah virus		MM 2021	[25 24 26 23]	[24 34 31 16]	[31 30 41 37]
		Republic of Korea	ROK-2.0017	[25 24 24 25]	[25 33 29 18] [26 32 29 18]	[31 31 40 37]
	Semliki forest virus			[28 23 26 21]	[DNP]	[35 33 33 38]
	Mayaro virus	Brazil	BeH256	[31 24 22 21]	[26 33 25 21]	[36 30 35 38]
	Ndumu virus	South Africa	SA AR221D	[27 26 21 24]	[DNP]	[32 35 32 40]
	Middelburg virus	South Africa	SaAr 749	[27 27 22 22]	[DNP]	[39 33 33 34]
	Trocara virus	Peru	PE 70009	[26 20 24 28]	[DNP]	[33 34 35 37]
	Barmah forest virus	Australia	BH2193	[30 23 23 22]	[DNP]	[29 39 28 43]
	Internal positive control			[23 24 25 21]	[25 33 26 16]	[36 30 36 32]

Within each column, identical base compositions for different isolates for a particular primer pair are shown grouped by the same color. DNP (did not prime) indicates that an RT-PCR amplicon was not be generated using the specified primer pair and virus. The numbers in brackets in columns under primer number indicate the numbers of each base (A, G, C, and T) generated from the target virus.

the subtype levels. All the different VEEV complex subtypes were resolved from one another with the exception of the IC and ID subtypes (which are not monophyletic). The sensitivity of each RT-PCR primer pair was determined using known quantities of internal positive control RNA. Fig. 2 shows the sensitivity of the each of the three primer pairs used in this study as a function of input RNA. The assay panel described here demonstrates, for the first time, a broad-priming approach using multiple, parallel detections that allows the rapid identification of all known alphaviruses at species or strain levels.

Detection of alphaviruses in laboratory-infected mosquitoes

To test the ability of the assay to detect alphavirus in RNA extracts obtained from a laboratory-infected mosquito in the presence of a large number of non-infected mosquitoes, four

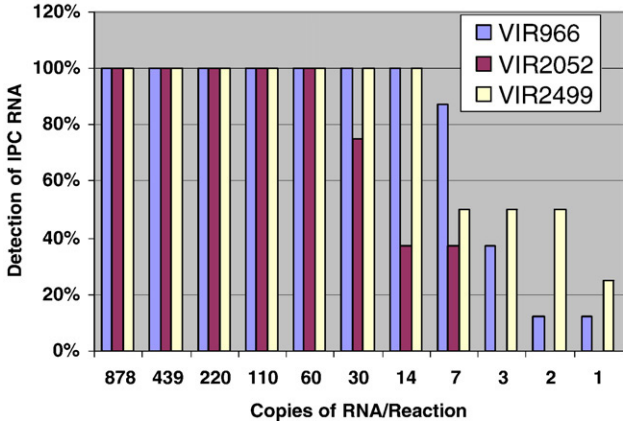

Fig. 2. Sensitivity of RT-PCR primer pairs VIR966, VIR2052, and VIR2499 when amplifying a known amount of internal positive control (IPC) RNA. The RNA control was produced *in vitro* and the copy number estimated from $O.D._{260}$ and the predicted molecular weight of the expected RNA transcript. Eight replicate detections were performed for each dilution and a score of 100% indicates that all reactions detected the IPC RNA.

separate pools of mosquitoes were prepared: a pool of 25 mosquitoes containing a single VEEV-infected mosquito (subtype IE 68U201); a pool of 50 mosquitoes containing a single VEEV-infected mosquito (subtype IE 68U201); a pool of 25 mosquitoes containing a single EEEV-infected mosquito (PE-0.0155); and a pool of 50 mosquitoes containing a single EEEV-infected mosquito (PE-0.0155). As shown in Table 4, VEEV and EEEV viruses were detected when RT-PCR was performed on extracted RNA from pools containing infected mosquitoes. When extracted RNA from pools containing EEEV-infected and VEEV-infected mosquitoes was mixed, both alphaviruses were readily detected using primers pairs VIR966 and VIR2499. Primer pair VIR2052, which does not amplify the PE-0.0155 strain of EEEV, detected only VEEV in the mixed sample. Fig. 3 shows the mass spectra of the RT-PCR amplicons generated from the mosquito pool containing both EEEV and VEEV. Panel A shows the clear detection of both amplicons by primer pair VIR966, primer pair VIR2499 also detects both viral amplicons. Though the amplitude for the VEEV amplicon is lower relative to the amplitude from the EEEV amplicon both amplicons are clearly detectable by our processor. As primer pair VIR2052 does not produce amplicons from EEEV, only the VEEV amplicon is produced and detected (panel C). This demonstrates the capability of the assay to detect and identify a mixture of two alphaviruses in a biologically relevant sample.

Detection of alphaviruses in field-collected mosquitoes and the identification of a member of the species Mucambo virus, VEE subtype IIID

Mosquitoes collected in the field provide an excellent means to monitor the environment for the presence of arboviruses and enables the early detection of emerging viruses. To test the ability of the assay to detect alphaviruses from field-collected mosquitoes, we assayed twelve pools of mosquitoes collected in Amazonian South America near Iquitos, Peru, and one pool of mosquitoes collected near Munson in the Republic of Korea. All had been previously tested and found to contain an alphavirus (Turell et al., 2005, 2003). Using RNA extracts from these mosquito pools, we found alphavirus in all of the samples (Table 5).

Two of the mosquito pools collected near Iquitos, Peru (PE-1.0800 and PE-2.1026), produced amplicons from only primer pair VIR2052. Una virus was the only alphavirus in our collection of isolates that was amplified by only this primer. Because the base composition signatures of the Una viruses in pools PE-1.0800 and PE-2.1026 were several mutations away from any previously tested or sequenced Una viruses, we could not confirm their identity beyond being alphaviruses using the assay RT-PCR ESI/MS assay. Sequence analysis of these isolates confirmed their identity as Una viruses. Mosquito pool ROK-2.0017, collected in the Republic of Korea, contained Getah virus. We were able to detect it with all three primer pairs. Primer pair VIR2499 produced two RT-PCR amplicons suggesting that this sample may contain a group of quasispecies or two distinct Getah viruses originating from different mosquitoes in the pool.

Three pools of mosquitoes (PE-40766, PE-40910, and PE-21.0185) collected near Iquitos, Peru, between January and February of 1997 contained an alphavirus that produced identical base composition signatures. Sequence analysis confirmed the relatedness of the mosquito pool viruses; the virus found in mosquito pools PE-40766 and PE-40910 are identical for 1477nt obtained using the three sequencing primers shown in Table 1. Due to limited sample RNA, only two of the three regions were sequenced from the virus found in mosquito pool PE-21.0185 (962nt) and this sequence was identical to the virus found in and PE-40766 except at a single nucleotide. To confirmed the identity of these viruses as a *Mucambo IIID* virus, we sequenced the three loci from the recently characterized *Mucambo IIID* virus (isolate FSL190, GenBank accession numberDQ228210) isolated from a patient in Iquitos, Peru (Aguilar et al., 2004). This sequence analysis of 1477nt obtained

Table 4
Detection of VEEV virus type IE strain 68U201 and EEEV virus strain PE-0.0155 from infected mosquitoes in pools of non-infected pools of mosquitoes

Sample	Type	Primer pair VIR966	Primer pair VIR2499	Primer pair VIR2052
Mosquito control	Negative	ND	ND	ND
Mosquito control	Negative	ND	ND	ND
Mosquito control	Negative	ND	ND	ND
Pool of 1:24 infected	EEEV	[27 25 25 21]	[24 34 26 21]	ND
Pool of 1:49 infected	EEEV	[27 25 25 21]	[24 34 26 21]	ND
Pool of 1:24 infected	VEEV	[25 27 22 24]	[27 34 26 18]	[34 34 32 39]
Pool of 1:49 infected	VEEV	[25 27 22 24]	[27 34 26 18]	[34 34 32 39]
Pool 2/98 mixture	EEEV and VEEV	[27 25 25 21] [25 27 22 24]	[24 34 26 21] [27 34 26 18]	[34 34 32 39]

RNA extracts from the 1:49 mixture of EEEV-infected mosquitoes and the 1:49 mixture VEEV-infected mosquitoes were mixed and assayed. The numbers in primer columns indicate the number of A, G, C, and T of the RT-PCR amplicons generated from the target virus. ND indicates no detection.

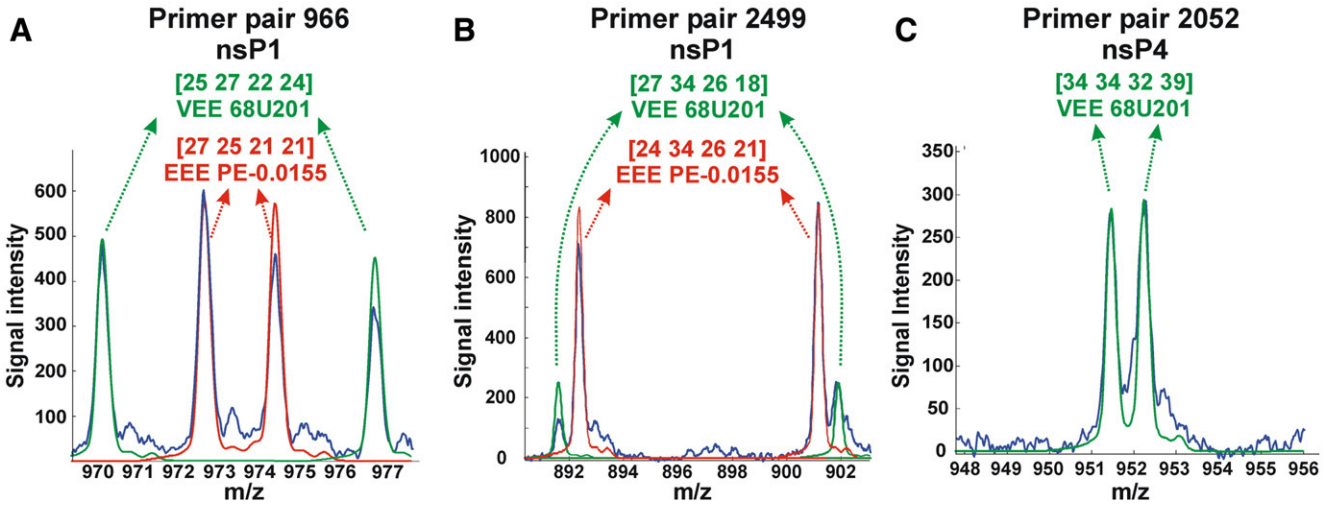

Fig. 3. Mass spectra of RT-PCR amplicons derived from RNA isolated from pools of mosquitoes consisting of 98 non-infected mosquitoes and one VEEV type IE strain 68U201-infected mosquito and one EEE strain PE-0.0155-infected mosquito. Primer pairs VIR966 (Panel A) and VIR2499 (Panel B) simultaneously detect and differentiate EEE and VEE viruses. Primer pair VIR2052 (Panel C) is specific to VEEV and does not amplify EEE strains. Paired peaks correspond to the sense and antisense strands of the RT-PCR amplicons, which separate under the conditions of electrospray ionization. Labels and peaks are colored according to detected organism: red = EEEV, green = VEEV. For each detected amplicon, theoretical spectra corresponding to sense and antisense amplicon strands (red and green traces) were overlaid upon the raw spectral data (blue trace) and one representative charge state for each product is shown.

using our three sequencing primers showed that there are only six nucleotide differences between FSL190 and the viruses found in mosquito pools PE-40766 and PE-40910. Fig. 4 shows the relationship of the Mucambo IIID viruses and other members of the VEEV complex.

Discussion

Alphaviruses are genetically highly diverse (Weston et al., 2005) creating challenges for their detection and monitoring in environmental samples such as RNA extracts from mosquitoes. Due to the diversity of alphavirus, dozens of separate assays must be performed to insure their detection. The possibility of more than one alphavirus type/species being present in a pool of mosquitoes presents additional challenges. We have developed a rapid, high-throughput assay using a very small set of universal primers that can detect and identify members of both New and Old World alphaviruses in a single assay. Additionally, the assay was able to simultaneously detect and identify multiple viral species in a single mixed sample. This capability is especially important in situations where there is the need to monitor samples where a large number of possible alphaviruses may be present.

The assay developed in this study was performed on the T-5000 system (Ibis Biosciences, Carlsbad, CA). The T-5000 system performs the de-salting and the mass spectrometry of the

Table 5
Detection of infected mosquitoes in RNA extracts from pools of mosquitoes collected in South America

Collection date	Collection location	Pool ID	Mosquito species	RT-PCR ESI/MS detection	VIR966	VIR02052	VIR2499
December 1996	Near Iquitos, Peru	PE-3.0609	*Cx. (Mel.) pedroi*	VEEV ID	[25 25 25 23]	[40 34 32 33]	[29 32 27 17]
September 1996	Near Iquitos, Peru	PE-1.0800	*Ps. ferox*	Alphavirus present	[DNP]	[44 29 31 35]	[DNP]
January 1997	Near Iquitos, Peru	PE-4.0766	*Cx. (Mel.) vomerifer*	VEEV-Like	[27 23 23 25]	[38 30 30 41]	[27 32 28 18]
October 1996	Near Iquitos, Peru	PE-2.1026	*Ps. ferox*	Alphavirus present	[DNP]	[45 28 32 34]	[DNP]
December 2000	Near Iquitos, Peru	PE-24.0269	*Cx. (Mel.) gnomatos*	VEEV IIIC	[26 25 24 23]	[38 27 34 40] (A > T)	[26 32 28 19]
January 1997	Near Iquitos, Peru	PE-4.0910	*Cx. (Mel.) pedroi*	VEEV-Like	[27 23 23 25]	[38 30 30 41]	[27 32 28 18]
December 1996	Near Iquitos, Peru	PE-3.0869	*Cx. (Mel.) pedroi*	EEEV	[27 25 25 21]	[DNP]	[24 34 26 21]
September 1996	Near Iquitos, Peru	PE-1.0433	*Ae. (Och.) hastatus*	WEEV	[26 27 23 22]	[36 34 33 36] (G > A)	[28 33 23 21]
January 1997	Near Iquitos, Peru	PE-4.0807	*Cx. (Mel.) pedroi*	EEEV	[26 24 27 21]	[43 31 30 35]	[24 34 25 22]
November 1999	Near Iquitos, Peru	PE-21.0185	*Cx. (Mel.) gnomatos*	VEEV-Like	[27 23 23 25]	[38 30 30 41]	[27 32 28 18]
February 1997	Near Iquitos, Peru	PE-5.0151	*Cx. (Mel.) spp.*	EEEV	[27 25 25 21]	[DNP]	[24 34 26 21]
October 2000	Munso, ROK	ROK-2.0017	*Ae. (Aed.) vexans*	Getah virus	[25 24 24 25] (T > C)	[31 31 40 37]	[25 33 29 18] [26 32 29 18]
February 2000	Near Iquitos, Peru	PE-22.0110	*Cx. (Mel.) pedroi*	EEEV	[27 25 25 21]	[DNP]	[24 34 26 21]

The virus from pool PE-40766 produced a signature that was distinct from that of all other alphaviruses. Sequencing of 1477 nt of the virus showed that it represented a novel subtype of the *Mucambo virus* species. The identification of single nucleotide polymorphisms from reference collection isolates is indicated in parentheses next to the base count signatures.

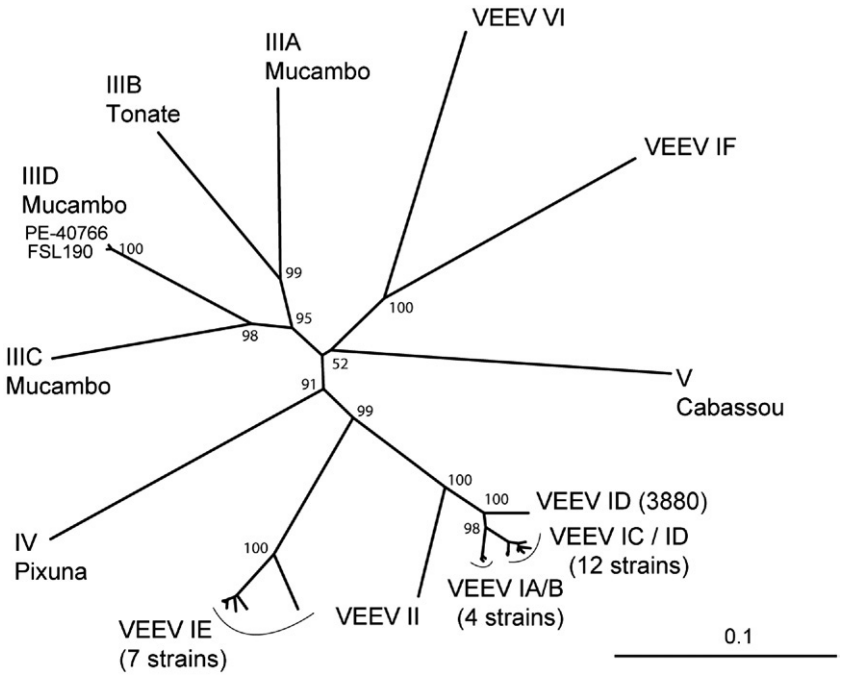

Fig. 4. Phylogenetic relationship of *Mucambo IIID* virus to other viruses in the VEEV complex based upon the concatenated alignment of 1477 nt for three different loci. The sequences for the IIID viruses were experimentally obtained using the three sequencing primer pairs listed in Table 1. The numbers at the branch points represent the bootstrap values.

samples containing RT-PCR amplicons and has the capacity to process over 400 samples in a 24-h period. The assay described here consists of three separate RT-PCR reactions.

Primer pair VIR966 can prime all but one of the alphaviruses tested and, by itself, can resolve 25 of the 34 viruses in the collection (Table 2). Primer pair VIR2499 contains inosines at several positions to facilitate broad-range priming. This pair primes all the New World alphaviruses tested but detected only 3 of the 10 Old World viruses. As each RT-PCR reaction contains an RNA internal positive control, RT-PCR reactions that fail to detect a virus can be readily distinguished from reactions that fail due to inhibitors or other causes. A third primer pair, VIR2052, was added to the set to allow detection of Chikungunya and other Old World alphaviruses. This primer pair also detected many of the New World alphaviruses in the collection, further improving the resolving power of the assay. This added resolution was particularly valuable for WEEV and to distinguish VEEV subtype IA/B from subtypes IC and ID.

The sensitivity of the assay was demonstrated using single VEEV- and EEEV-infected mosquitoes in pools of 25 or 50 uninfected mosquitoes and in a mixture for VEEV- and EEEV-infected mosquito pools. RNA was extracted from mosquitoes and viral RNA was detected from all pools containing an infected mosquito(s). We were also able to detect and identify virus in RNA extracts from pools of naturally infected mosquitoes obtained from Amazonian South America and the Republic of Korea. Mosquitoes collected from these locations were found to be infected with VEEV ID, *Mucambo IIID* virus species, EEEV, Getah virus, Una, and WEEV.

Our detection of a *Mucambo IIID* virus demonstrates the ability of the broad-range assay to detect novel alphaviruses where little or no sequence information is available. This capability is critical for surveillance of emerging infectious pathogens and bio-warfare agents.

Materials and methods

Virus isolates

The viral strains used in this study were supplied by the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) or the University of Texas Medical Branch (Table 1). The viruses from the USAMRIID collection were plaque purified in Vero-76 cells and amplified in BHK-21 cells. BHK-21 cells were infected at high (3) or low (0.01) multiplicities of infection for the production of large-volume, high-titer-infected cell culture supernatants for virus purification. Viruses were purified from infected cell culture supernatants by sucrose density centrifugation. The purity of the virus preparations was determined by polyacrylamide gel electrophoresis and colloidal blue staining and only pure preparations with the predicted protein profiles were used to prepare RNA. The University of Texas Medical Branch viruses were plaque purified and virus extracted directly from the cultured cells and supernatants.

Mosquito inoculation

Aedes (*Ochlerotatus*) *taeniorhynchus* mosquitoes were inoculated intrathoracically (Rosen and Gubler, 1974) with 0.3 μL of a suspension containing $10^{4.8}$ PFU/mL ($10^{1.3}$ PFU inoculated per mosquito) of EEEV (strain PE-0.0155), originally

isolated from *Culex pedroi* mosquitoes captured in Peru in 1996 (Turell et al., 2005), or subtype IE of VEEV (68U201), originally isolated from a hamster in Guatemala in 1968 (Scherer et al., 1970). After inoculation, mosquitoes were held in cardboard containers in an incubator maintained at 26 °C with a 16:8 L:D light cycle and high humidity until tested for virus 7 days after inoculation. Previous studies indicated that these mosquitoes would contain about $10^{6.5}$ PFU/mosquito after 7 days. Mosquitoes were provided apple slices daily as a carbohydrate source.

Extraction of viral RNA

RNA from the USAMRIID viral RNA panel was isolated from sucrose purified virus preparations by TRIzol LS (Invitrogen, Carlsbad, CA) extraction following the manufacturer's instructions. RNA was quantified and checked for quality by measuring absorbance at 260 and 280 nm. Isolation of full-length genomic RNA was confirmed by electrophoresis in denaturing formaldehyde gels and visualization by ethidium bromide staining. The RNA was reverse transcribed using poly dT with genus- or strain-specific primers. The identity of each RNA standard was confirmed by sequence analysis of the RT-PCR products and comparison with published data.

Viral RNA from pooled mosquitoes and the University of Texas Medical Branch were extracted by using TRIzol LC (Invitrogen Carlsbad, CA) in combination with the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA). Briefly, TRIzol LC was added in a 3:1 ratio to pooled mosquito homogenates and stored at −70 °C until RNA extraction. Viral RNA was extracted by the addition 2.5 μg of sheared poly-A RNA carrier (Qiagen, Chatsworth, CA) and 0.1 mL of chloroform to 0.5 mL of the serum/extract TRIzol mixture. The mixture was incubated at room temperature for 15 min and centrifuged at 12,000×*g* in a bench-top microcentrifuge. The resulting, clear, top layer (400 μL) was transferred to a new tube and one volume of 70% ethanol was added to the sample. The entire volume was transferred to an RNeasy Mini Spin Column in a 2-mL collection tube. The tube was centrifuged for 15 s at 8000×*g* and the column was washed once with 500 μL RPE buffer and 1× with 80% ethanol. The viral RNA was subsequently eluted in 40 μL RNAse-free water into a tube containing 1 μL of Superasin (Ambion).

Primer design

Several primer pairs were designed to broadly target various alphavirus species. As an example (Fig. 1), the primer sequence for primer pair VIR966 (Table 1) is shown overlaid on an alignment of available nsp1 viral sequences, with the dots indicating identity to the primer sequence. Three primer pairs shown in the top half of Table 1 were selected for use in the final assay based on their breadth of coverage of various alphavirus isolates and ability to resolve the different species from each other (VIR966, VIR2499, and VIR2052). Primer pair VIR2499 included modified inosines at several locations (five inosines in the forward primer and three in the reverse) in order to overcome sequence diversity in the primer region. All primers used in this study had a thymine nucleotide at the 5′-end to minimize addition of non-templated adenosines during amplification using Taq polymerase (Brownstein et al., 1996).

RT-PCR

One-step RT-PCR was performed in a reaction mix consisting of 4 U of Ampli*Taq* Gold (Applied Biossytems, Foster City, CA), 20 mM Tris (pH 8.3), 75 mM KCl, 1.5 mM $MgCl_2$, 0.4 M betaine, 200 μM dATP, 200 μM dCTP, 200 μM dTTP (each dNTP from Bioline USA, Randolph, MA), and 200 μM ^{13}C-enriched dGTP (Spectra Stable Isotopes, Columbia, MD), 10 mM dithiothreitol, 100 ng sonicated poly-A DNA (Sigma Corp, St Louis MO), 40 ng random primers (Invitrogen Corp, Carlsbad, CA.), 1.2 U Superasin (Ambion Corp, Austin TX), 400 ng T4 gene 32 protein (Roche Diagnostics Corp., Indianapolis, IN), 2 U Superscript III (Invitrogen Corp, Carlsbad, CA.), 20 mM sorbitol (Sigma Corp, St Louis, MO), and 250 nM of each primer. The following RT-PCR cycling conditions were used: 60 °C for 5 min, 4 °C for 10 min, 55 °C for 45 min, 95 °C for 10 min, followed by 8 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C 30 s, with the 48 °C annealing temperature increasing 0.9 °C each cycle. The PCR was then continued for 37 additional cycles of 95 °C for 15 s, 56 °C for 20 s, and 72 °C for 20 s. The RT-PCR cycle ended with a final extension of 2 min at 72 °C followed by a 4 °C hold.

Sequencing of Novel Virus IIID virus

Sequencing of the IIID virus was performed using the three sequencing primer pairs shown in the lower half of Table 1. The reactions were performed using the above described RT-PCR reaction conditions with the following RT-PCR cycle: 60 °C for 5 min, 4 °C for 10 min, 55 °C for 45 min, 95 °C for 10 min, followed by 8 cycles of 95 °C for 15 s, 50 °C for 45 s, and 72 °C 90 s with the 50 °C annealing temperature increasing 0.6 °C each cycle. The PCR was then continued for 37 additional cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 60 s. The RT-PCR cycle ended with a final extension of 4 min at 72 °C followed by a 4 °C hold. All RT-PCR reactions were performed using MJ Thermocyclers (BioRad, Hercules, CA). Resulting amplicons were sequenced using M13 primers by Retrogen Inc. (San Diego, CA).

Internal positive control RNA

An internal positive control (IPC) made from synthetic RNA was included in each RT-PCR at an experimentally determined concentration (30 copies). The IPC was produced by *in vitro* transcription from a T7 promoter present on cloned synthetic DNA template containing the target regions for primer pairs VIR966, VIR2499, and VIR2052. VEEV strain 3908 subtype IC (GenBank index 20800454) sequence was used for the target regions for primer pairs VIR966 and VIR2499. Primer pair VIR2052 targets the Old World alphaviruses and was based on Chikungunya virus sequence (GenBank index 27754751). The

T7 promoter is oriented such that the RNA transcript produced is the same sense strand as alphavirus RNA. Following *in vitro* transcription using the Megascript T7 kit (Ambion, Austin TX) according the manufacturer's instructions, the DNA template was digested by the addition of 50 U of DNAse I (Roche, Indianapolis, IN), 14 μL water, 1 μL of Superasin (Ambion), and incubation at 37 °C for 1 h. Following digestion, the RNA was purified using the RNeasy RNA isolation kit (Qiagen, Chatsworth, CA) following the manufacturer's protocol. The IPC is designed to utilize the same PCR primer pairs used for alphavirus detection but generates an amplicon containing a 5-base pair deletion that can be clearly resolved from the viral amplicons.

Mass spectrometry and base composition analysis

Following amplification, 30 μL aliquots of each PCR reaction was desalted and purified using a weak anion exchange protocol as described elsewhere (Jiang and Hofstadler, 2003). Accurate mass (±1 ppm), high-resolution (M/dM >100,000 FWHM) mass spectra were acquired for each sample using high-throughput ESI-MS protocols described previously (Hofstadler et al., 2005). For each sample, approximately 1.5 μL of analyte solution was consumed during the 74-s spectral acquisition. Raw mass spectra were post-calibrated with an internal mass standard and deconvolved to monoisotopic molecular masses. Unambiguous base compositions were derived from the exact mass measurements of the complementary single-stranded oligonucleotides (Muddiman et al., 1997). Quantitative results are obtained by comparing the peak heights with an IPC present in every PCR well at 100 molecules (Hofstadler et al., 2005).

Phylogenetic analysis

Neighbor-joining trees were constructed using the dnadist and neighbor programs of the Phylip package (http://evolution.genetics.washington.edu/phylip.html). The VEEV tree was assembled using our novel VEEV sequence and publicly available sequences fragments representing the different VEEV subtypes. The trees were bootstrapped using the seqboot and consense programs of the Phylip package and were analyzed with treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The following GenBank sequences (gi) were used in the phylogenic analysis: VEEV IA/B, 323714, 53680681, 323708, 3249013; VEEV IC ID, 20800448, 20800451, 20800454, 290609, 5442464, 5442468, 14549692, 18152933, 5442458, 5442461, 4689187, 5442471,323706; VEEV II,4262299; VEEV IE, 4262302,17865005, 1144527, 56789993, 17864993, 17864996, 17864999,17865002; VEEV IIIA,4262305; VEEV IIIB,4262308; VEEV IIIC,4262311; VEEV IV,4262314; VEEV V,4262323; VEEV IF,4262317; VEEV VI,4262320.

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